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Advances in Brief

Intestinal Neoplasia in the *Apc*^{Min} Mouse: Independence from the Microbial and Natural Killer (*beige* Locus) Status[1]

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Abstract

We have tested the hypothesis that enteric bacteria are necessary for formation of intestinal adenomas in C57BL/6-*Apc*^{Min}/+ mouse. Germ-free mice developed 2-fold fewer adenomas than conventional controls in the medial small intestine (7.3 *versus* 14.9; $P < 0.003$), but there were no significant differences in the rest of the intestinal tract. We conclude that microbial status does not strongly alter the adenoma phenotype in this mouse model of familial adenomatous polyposis. In parallel, we have found that C57BL/6-*Apc*^{Min}/+ mice mutated at the *beige* locus, which controls natural killer activity, are also unaltered in adenoma multiplicity.

Introduction

Gastrointestinal cancer in human populations is affected by both genetic and environmental variables. The role of some of these can be analyzed by controlled comparisons when an appropriate animal model offers genetic homogeneity and a controllable environment. The congenic Min[6] mouse strain models at least the early steps of familial adenomatous polyposis in the human (1, 2). Through use of this strain, a steadily increasing number of genetic and environmental factors have been shown to affect the multiplicity and/or net growth rate of intestinal adenomas (3). It is possible *a priori* that some of these risk-modifying factors operate by influencing the microbial ecosystem of the intestinal tract or by stimulating or suppressing the set of lymphoid cells commonly classified as NK cells. In the human, the microflora is confined to the distal small intestine and the colon (4). In rodents and the pig, however, bacterial species are abundant in all regions of the intestinal tract (4). Interestingly, tumors and microflora are distributed similarly: Min mice develop adenomas throughout the intestinal tract (1), whereas familial adenomatous polyposis in the human predominantly affects the colon. In this report, we present a study of the incidence of intestinal neoplasia when Min mice are either germ-free or lack the NK activity controlled by *bg* (5).

Materials and Methods

Mice. Min animals were bred in our mouse colony at the McArdle Laboratory for Cancer Research, with C57BL/6J from The Jackson Laboratory (Bar Harbor, ME) as the background strain for continuing backcrosses from *Min*/+ males. Teklad Mouse Breeder Diet 8626 (Harlan-Teklad, Madison, WI) and automatically supplied water (acidified to pH 2.6) were provided *ad libitum* in production breeding and in the crosses that generated C57BL/6-*bg*^{J}/*bg*^{J} homozygotes.

Mice for the germ-free experiments and their conventionally raised controls were obtained by caesarian and normal delivery, respectively. Pregnant mothers were transferred from the McArdle colony to the Gnotobiote Laboratory on the 18th day of gestation. The germ-free animals were derived by sterile hysterectomy on that day and fostered to parous BALB/c mothers in a sterile microisolator. The conventional set, by contrast, was permitted to deliver normally and was caged within the same facility but outside sterile containment. Animals carrying the *Min* mutation were genotyped as described previously (6). Feed for both the germ-free and conventional animals was Purina 5010 Autoclavable Rodent Chow (Purina Mills, Richmond, IN), autoclaved at 250°F for 30 min and dried for 15 min. Similarly, water for both sets of animals was prepared in common by reverse osmosis and autoclaving. The bedding for both sets was ¼ inch of corncob fragments, also autoclaved.

Germ-free Monitoring. A comprehensive mouse serology panel was run for the germ-free animals by the University of Missouri Research Animal Diagnostic and Investigative Laboratory and was found to be negative for all agents tested: mouse hepatitus virus, Sendai virus, pneumonia virus of mice, reovirus-3, Theiler's murine encephalomyelitis virus, ectromelia virus, mouse adenovirus-1, mouse adenovirus-2, Polyomavirus, *Mycoplasma pulmonis*, lymphocytic choriomeningitis virus, epizootic diarrhea of infant mice virus, *Encephalitozoon cuniculi*, cilia-associated respiratory bacillus, and Parvovirus. The bacterial status was monitored weekly by exposing a pair of sterile cotton swabs to the water, the cage lids, and a fresh fecal sample. One swab was then cultured for a week in Remel's Blood Agar and Thioglycolate Broth at 37°C for aerobes, and the other for a week in anaerobic blood agar at 37°C for anaerobes. No bacterial growth was detected throughout the course of the germ-free experiment, and Gram strains of fecal smears confirmed the absence of bacteria. By contrast, our conventional animals were found to be positive for *Parvovirus*, mouse papule virus, *Helicobacter hepaticus*, and (for the experiment in Table 3) mouse hepatitis virus. Fecal smears from the conventional animals show a broad range of Gram-positive and Gram-negative bacteria.

Cross with *beige*. C57BL/6-*bg*^{J}/*bg*^{J} homozygotes from The Jackson Laboratory were crossed with C57BL/6-*Min*/+ to produce *bg*^{J}/+*Min*/+ double heterozygotes. These were then backcrossed to the *bg*^{J}/*bg*^{J} homozygotes to produce an N2 population in which animals carrying *Min* were identified by the presence of intestinal tumors. *bg*^{J}/*bg*^{J} homozygotes were distinguished from *bg*^{J}/+ heterozygotes by coat color.

Tumor Scoring and Size Determination. For the germ-free and conventional sets of Min animals, tumors were scored after formalin fixation and methylene blue staining of 4-cm sections of the proximal, medial, and distal small intestine and entire colon as described previously (6). For the *beige* cross, the scoring was performed as described previously (1). Tumor size distributions were determined after formalin fixation by registering the maximum diameter under a Nikon SMZ/U stereomicroscope for each adenoma of the small intestine. Adenomas of the intestine were validated histologically as described previously (6, 7). Because observers differ in their ability to detect adenomas, all comparisons between groups of animals are unified by observer.

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[6] The abbreviations used are: Min mouse, C57BL/6-*Apc*^{Min}/+; NK, natural killer; *bg*, *beige* locus; *Min*, multiple intestinal neoplasia; *Apc*, adenomatous polyposis coli; CV, coefficient of variation.

Results

The tumor multiplicities of 12 conventional and 13 germ-free Min animals are summarized in Table 1. In each case, counts were determined by three independent observers, for one of whom the samples were encoded and randomized, to prevent subjective bias. The observers did not differ systematically from one another, showing a CV for individual samples ranging from 13–31% (see Table 1).

The most significant effect of microbial status on tumor multiplicity is found in the medial section of the small intestine, where germ-free animals developed an average of 7.3 adenomas in the counted region, whereas conventional animals developed an average of 14.9 ($P = 0.003$). The fact that the conventional animals were slightly younger at sacrifice accentuates the significance of this difference. The colon was analyzed with particular care because of the report by Gould and Dove (8) that sterile ectopic grafts of fetal colonic tissue fail to develop adenomas. For any lesion that was in question, a sample was prepared for histological examination (see Ref. 7). Only those explicitly shown to be adenomas were included in Table 1. The germ-free animals developed an average of 0.69 adenomas in the colon, whereas their conventional counterparts developed an average of 1.1 ($P = 0.36$).

Tumors in the small intestine are not pedunculated. This permits facile scoring of their maximum diameters. Table 2 presents the measurements of two observers. For both the medial and the distal segments of the small intestine, the tumors in the germ-free animals were significantly larger than those in the conventional animals ($P = 0.001$). The small age difference between the germ-free and the conventional animals provides a simple explanation for this difference. Beyond this, the cecum of an animal raised under germ-free conditions is markedly enlarged, leading to distention of the small intestine and conceivably contributing to the enhancement of tumor size observed in portions of the small intestine of germ-free Min animals.

In a separate analysis carried out entirely within the McArdle colony, we analyzed Min mice that were either heterozygous or homozygous for the bg^J mutation and so deficient in NK activity. Tumors were counted as described previously (1). The totals are tabulated in Table 3. There is no significant difference in total tumor multiplicity or regional distribution between the two genotypic classes at *bg*.

Discussion

The possibility that the microbial flora of the intestinal tract can affect the incidence and/or progression of neoplasms is more than speculative. Several decades ago, Laqueur *et al.* (9) demonstrated that cycasin was ineffectual as an intestinal carcinogen in germ-free rats in contrast to conventional controls. Bacterial species elaborate hydrolases that release the active carcinogen, methylazoxymethanol, from its β-glucoside cycasin. More recently, prospective epidemiological studies in the human have shown significant association between *H. pylori* infection and subsequent gastric adenocarcinoma (10). Falk *et al.* (11) have shown that *H. pylori* adheres specifically to surface mucous cells of the gastric epithelium. Although *H. pylori* has not yet been rigorously proven to be the causative agent for the associations with neoplastic lesions, it certainly is possible either that these bacterial populations are responsible for the production of carcinogens or promoting agents, or else that local lesions caused by bacterial infection provoke a neoplastic process.

In the mouse, intestinal whole mounts from germ-free animals have been shown to lack the cell surface pattern of fucosylation detected by lectin binding on such preparations from conventional mice (12). In this situation, the reintroduction of a single bacterial species, *Bacteroides thetaiotaomicron*, reactivates the expression of the *fucosyl transferase* gene needed to establish the pattern of fucosylation on the intestinal enterocytes. If microbial flora can influence the cell biology of the intestinal epithelium, perhaps it can also affect the neoplastic transformations of the intestinal epithelium. In particular, a major resistance modifier allele affecting tumor multiplicity in the Min mouse, *Mom1* (13), may encode a secreted phospholipase (14)[7] known to possess bactericidal activity (15).

Grafts of segments of intestine from fetal donors under the dorsal skin of adult mouse recipients persist for several months, permitting tumors to form if the donor tissue is *Min*/+ (8). Although donor tissue from the small intestine develops adenomas at a higher frequency than expected from normal intestine, that from fetal colon fails to develop adenomas. These ectopic grafts remain sterile throughout the duration of the experiment, prompting the hypothesis that the gut flora provides a nec-

Table 1 *Tumor counts for germ-free Min mice*

Thirteen germ-free mice ages 101 ± 10 days and 12 conventional mice ages 85 ± 1 days were examined by three independent observers, one fully blind as to the history of the samples.

Location	Average ± SD (CV[a]) Germ-free	Conventional	*P* value[b]
Small intestine[c]			
Proximal	7.15 ± 4.72 (0.28)	4.92 ± 3.94 (0.19)	0.191
Medial	7.27 ± 7.00 (0.31)[d]	14.92 ± 5.11 (0.18)	0.003
Distal	12.77 ± 5.72 (0.13)	10.75 ± 7.30 (0.21)[e]	0.313
Colon[f]	0.69 ± 0.75	1.08 ± 1.00	0.361
Total	27.88 ± 13.13	31.67 ± 9.51	0.310

[a] Computed as the average of the individual CVs for each tissue. Each individual CV is the SD across observers divided by the mean count for that sample.
[b] Exact significance level computed in a two-sided randomization test for the *t* statistic of square-root transformed counts, using $10^4 - 1$ simulated allocations.
[c] Calculations based on the median of the counts by the three observers.
[d] One sample was not counted by one of the observers.
[e] One sample was not counted by two of the observers.
[f] Calculations based on the consensus of counts of the three observers, using histological analysis in cases of discrepancies.

Table 2 *Sizes of tumors in germ-free Min mice*

Tumors were measured by two observers in 13 germ-free mice ages 101 ± 10 days and 12 conventional mice ages 85 ± 1 days.

Section of small intestine	Average of maximum diameter (mm) ± SD[a] Germ-free	Conventional	*P* value[b]
Proximal	1.53 ± 0.29	1.70 ± 0.52	0.17
Medial	1.27 ± 0.32	0.85 ± 0.14	<0.001
Distal	1.23 ± 0.30	0.84 ± 0.13	<0.001

[a] For each observer, the maximum tumor diameters were averaged across tumors in each tissue sample. The results from the two observers were then averaged. These observer-corrected mean maximum diameters were then averaged over all tissue samples in each group. The resulting means and raw SDs are reported. To assess variation across observers, relative errors were calculated for each tissue sample. On average over samples, this relative error was 13%.
[b] Calculated by comparing a weighted two-sample *t* statistic with its randomization distribution by use of $10^4 - 1$ simulated allocations. Sample measurements were weighted by the number of tumors in the sample to improve precision. Separate analyses of the data from each observer yielded equivalent results.

Table 3 *Tumor counts in Min mice carrying beige*

Tumors were counted by one observer in 15 bg^J/bg^J mice ages 95 ± 10 days and 27 $bg^J/+$ mice ages 98 ± 11 days.

Location	Average ± SD bg^J/bg^J	$bg^J/+$	*P* value[a]
Small intestine			
Proximal	7.53 ± 3.11	6.74 ± 3.19	0.44
Medial	7.47 ± 3.89	8.85 ± 5.23	0.38
Distal	3.47 ± 3.48	3.41 ± 3.10	0.95
Colon	4.20 ± 2.34	3.41 ± 3.47	0.44
Total	22.67 ± 7.02	22.41 ± 9.04	0.92

[a] Calculated by separate two-sided two-sample *t* tests.

[7] K. A. Gould, C. Luongo, A. R. Moser, M. K. McNeley, N. Borenstein, A. Shedlovsky, W. F. Dove, K. Hong, W. F. Dietrich, and E. S. Lander. Genetic evaluation of candidate genes for the *Mom1* modifier of intestinal neoplasia in mice, Genetics, *144:* 1777–1785, 1996.

essary element in the neoplastic process, at least in the *Min*/+ colon. This hypothesis has been tested by the experiment summarized in Tables 1 and 2.

The microbial status of Min mice does not strongly alter the adenoma multiplicity in either the small or large intestine (Table 1). The results for the colon indicate that the maximum likelihood effect of germ-free status, calculated on a Poisson model, is a small reduction of multiplicity to 0.64 of control values (95% confidence interval, 0.26–1.48). The failure to observe adenomas from grafted *Min*/+ fetal intestine involved an amount of tissue for which 13 tumors were expected on the null hypothesis (8). If germ-free status reduces tumor multiplicity to 0.26 of control (the 95% confidence limit), one would have expected 3.4 tumors in this set of ectopic colonic grafts. Observing zero tumors is significant evidence that the nonautonomy of *Min*-induced tumor formation for the grafted colon involves factors beyond any effect of the microbial flora ($P = 0.03$). Clearly, experiments larger in scale are necessary to measure any subtle quantitative effect on colonic tumor formation by gut microbes. At this point, we limit our major conclusion to the finding that the microbial flora is not necessary for adenoma formation in either the large or small intestine of the Min mouse.

Quantitatively, the most significant effect of the germ-free status is the decrease in tumor multiplicity in the medial small intestine. Unlike the jejunum, the corresponding region in the human, which is deficient in microflora, the small intestine of the mouse carries an abundant population of bacteria (4). Though not necessary for adenoma formation or growth in the Min mouse, these microbes could enhance adenomagenesis by the production of endogenous carcinogens.

The microbial ecosystem of the gut not only is a possible source of mutagens, but also can interact with the immune system. Therefore, one must consider the multifaceted components of immunity in evaluating systemic factors that can affect intestinal tumor formation. Dudley *et al.* (16) have reported that homozygosity for the severe combined immunodeficiency mutation, *scid*, does not perceptibly affect the intestinal neoplastic phenotype of Min animals. Thus, any immune surveillance processes mediated by classical T-cell and B-cell mechanisms are not major barriers to intestinal neoplasia in the Min mouse.

In principle, discrimination by the organism between self and nonself can include processes other than those currently comprehended within the clonal selection paradigm for the T- and B-cell lineages. "Natural resistance" may involve a more generic discrimination of nonself not depending on the generation of diversity that is controlled by the *scid* locus (see Ref. 17). By contrast, at least some NK activities are lost in lymphoid cells from mice homozygous for the bg^J mutation (18). If adenoma formation involves the expression of molecular species that can be detected by NK cells, one might expect that adenoma formation would be more efficient in mice deficient in NK activity. The hypothesis that this mode of surveillance controls adenoma formation in the Min mouse has been tested in the experiment summarized in Table 3. Clearly, no evidence for such an effect has been found ($P = 0.92$).

Altogether, the investigations reported here indicate that the phenotype of the Min mouse is minimally affected by manipulations of the microbial flora or the system of natural resistance. In contrast to this robust behavior of the Min phenotype, adenoma multiplicity of this mouse model is reduced by up to a factor of 8 by appropriate doses of the nonsteroidal anti-inflammatory agent piroxicam (6). Beyond these environmental effects, it is intriguing to note that the multiplicity of colonic adenomas observed in the germ-free and conventional animals reported here (mean, 0.69–1.08; Table 1) is substantially lower than we observed in our standard McArdle Min colony (mean, 3.4; Table 3). There was also a substantial increase in the adenoma multiplicity of the distal region of the small intestine in both the conventional and the germ-free animals of Table 1 compared to those of Table 3. One possible explanation is the reduced fat content and higher fiber content of the Purina diet (6% and 4.4%, respectively) used in the germ-free experiments and their conventional controls, compared with the Teklad diet (≥10% fat and ≤2% fiber) used in our standard Min colony. Previous observations have indicated that there are dietary effects on the Min phenotype (6, 19). A separate possible cause for the difference between the tumor multiplicities of the conventional animals is a change in the microbial flora between the experiment in Table 3 and that in Table 1 when we eliminated mouse hepatitis virus from our colony by caesarian derivation. Though microbial flora in general is not necessary for the Min phenotype, it is possible that particular species can exert an effect.

Effects of diet and microbial status on the Min phenotype deserve further controlled study.

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References

1. Moser, A. R., Pitot, H. C., and Dove, W. F. A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. Science (Washington DC), *247:* 322–324, 1990.
2. Su, L-K., Kinzler, K. W., Vogelstein, B., Preisinger, A. C., Moser, A. R., Luongo, C., Gould, K. A., and Dove, W. F. A germline mutation of the murine homolog of the *APC* gene causes multiple intestinal neoplasia. Science (Washington DC), *256:* 668–670, 1992.
3. Shoemaker, A. R., Gould, K. A., Luongo, C., Moser, A. R., and Dove, W. F. Studies of neoplasia in the Min mouse. Biochim. Biophys. Acta Rev. Cancer on Line, *1332:* pf25–pf48, 1996.
4. Tannock, G. W. Normal Microflora. London: Chapman and Hall, Ltd., 1995.
5. Lane, P. W., and Murphy, E. D. Susceptibility to spontaneous pneumonitis in an inbred strain of beige and satin mice. Genetics, *72:* 451–460, 1972.
6. Jacoby, R. F., Marshall, D. J., Newton, M. A., Novakovic, K., Tutsch, K., Cole, C. E., Lubet, R. A., Kelloff, G. J., Verma, A., Moser, A. R., and Dove, W. F. Chemoprevention of spontaneous intestinal adenomas in the Apc^{Min} mouse model by the nonsteroidal anti-inflammatory drug piroxicam. Cancer Res., *56:* 710–714, 1996.
7. Shoemaker, A. R., Moser, A. R., and Dove, W. F. ENU treatment of Min mice: age-related effects on the formation of intestinal adenomas, cystic crypts, and epidermoid cysts. Cancer Res., *55:* 4479–4485, 1995.
8. Gould, K. A., and Dove, W. F. Action of *Min* and *Mom1* on neoplasia in ectopic intestinal grafts. Cell Growth & Differ., *7:* 1361–1368, 1996.
9. Laqueur, G. L., McDaniel, E. G., and Matsumoto, H. Tumor induction in germfree rats with methylazoxymethanol (MAM) and synthetic MAM acetate. J. Natl. Cancer Inst., *39:* 355–371, 1967.
10. Forman, D., Newell, D. G., Fullerton, F., Yarnell, J. W. G., Stacey, A. R., Wald, N., and Sitas, F. Association between infection with *Helicobacter pylori* and risk of gastric cancer: evidence from a prospective investigation. Br. Med. J., *302:* 1302–1305, 1991.
11. Falk, P., Roth, K. A., Borén, T., Westblom, T. U., Gordon, J. I., and Normark, S. An *in vitro* adherence assay reveals that *Helicobacter pylori* exhibits cell lineage-specific tropism in the human gastric epithelium. Proc. Natl. Acad. Sci. USA, *90:* 2035–2039, 1993.
12. Bry, L., Falk, P. G., Midtvedt, T., and Gordon, J. I. A model of host-microbial interactions in an open mammalian ecosystem. Science (Washington DC), *273:* 1380–1383, 1996.
13. Dietrich, W., Lander, E., Smith, J. S., Moser, A. R., Gould, K. A., Luongo, C., Borenstein, N., and Dove, W. F. Genetic identification of *Mom-1*, a major modifier locus affecting *Min*-induced intestinal neoplasia in the mouse. Cell, *75:* 631–639, 1993.
14. MacPhee, M., Chepenik, K. P., Liddell, R. A., Nelson, K. K., Siracusa, L. D., and Buchberg, A. M. The secretory phospholipase-a2 gene is a candidate for the *Mom1* locus, a major modifier of Apc^{Min}-induced intestinal neoplasia. Cell, *81:* 957–966, 1995.
15. Harwig, S. S. L., Tan, L., Qu, X-D., Cho, Y., Eisenhauer, P. B., and Lehrer, R. I. Bactericidal properties of murine intestinal phospholipase A_2. J. Clin. Invest., *95:* 603–610, 1995.
16. Dudley, M. E., Sundberg, J. P., and Roopenian, D. C. Frequency and histological appearance of adenomas in multiple intestinal neoplasia mice are unaffected by severe combined immunodeficiency (*scid*) mutation. Int. J. Cancer, *65:* 249–253, 1996.
17. Kaufman, D. S., Schoon, R. A., and Leibson, P. J. MHC class I expression on tumor targets inhibits natural killer cell-mediated cytotoxicity without interfering with target recognition. J. Immunol., *150:* 1429–1436, 1993.
18. Roder, J. C. The beige mutation in the mouse. I. A stem cell predetermined impairment in natural killer cell function. J. Immunol., *123:* 2168–2173, 1979.
19. Kim, S. H., Roth, K. A., Moser, A. R., and Gordon, J. I. Transgenic mouse models that explore the multistep hypothesis of intestinal neoplasia. J. Cell. Biol., *123:* 877–893, 1993.

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